FIELD OF THE TECHNIQUE

The present invention relates to a process for forming aggregates (associated products) of hydrophobic group-containing polysaccharides.

BACKGROUND OF THE TECHNIQUE

Hydrophobic group-containing high molecular weight polysaccharides in which hydrophobic group(s) are bound to a polysaccharide are used for medicinal materials, for example, coating material for coating a drug carrier enclosing therein a drug. It is known that, by coating a drug carrier, for example, a liposome microcapsule, microsphere, O/W emulsion or erythrocyte ghost, with a hydrophobic group-containing polysaccharide, not only the spontaneous exudation of a drug from such a drug carrier is suppressed but also the cell-specific drug transference rate using such a drug carrier is improved.

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It has in recent years been widely accepted that liposomes and O/W emulsions are prospective as drug carriers. It has been reported that the chemical and physical stabilities of a drug carrier of this kind within and without a living body are improved by coating the drug carrier with a polysaccharide, wherein thereby a target-tropism to a specific cell group is also revealed {Bull. Chem. Soc. Japan, 62, 791-796 (1989)}. It has further been reported that liposomes are physically stabilized by coating them with a polysaccharide {Drug Delivery System, 5, 261 (1990)}.

Further, it is reported that hydrophobic group-containing polysaccharides interact with proteins and with compounds exhibiting higher hydrophobicity so as to encapsulate these proteins or compounds {Chem. Lett., 1263 (1991)}. In this literature, it is described that, when aggregates of a hydrophobic group-containing polysaccharide are mixed with a globular protein of varying kinds at room temperature, the protein becomes coupled with the aggregates of the hydrophobic

group-containing polysaccharide to form a conjugate. Therein is also described that aggregates of hydrophobic group-containing polysaccharides are stable, even in the presence of excess amounts of such proteins.

Further, a vaccine product containing a hydrophobic group-containing polysaccharide and an antigen is also known (WO 98/09650). It is furthermore known that a conjugate of a hydrophobic group-containing polysaccharide and an antigen can be isolated and purified by mixing aggregates of the hydrophobic group-containing polysaccharide with the antigen at room temperature, and, then, treating the resulting mixture by gel chromatography {Macromolecules,. 7654 (1994)}.

On the other hand, Akiyoshi et al disclose in Macromolecules,. 3062 (1993) that hydrophobicized polymeric substances are subject to intra- or inter-molecular self association of their hydrophobic groups in a dilute aqueous solution, resulting in formation of aggregates of the polymer molecules. In particular, a hydrophobic group-containing polysaccharide forms relatively monodisperse microparticles of aggregates in a size of nano-order in a dilute aqueous solution by spontaneous association of several molecules. It is confirmed by observation under electron microscope that relatively monodisperse globular microparticles of nano-order size are formed. Such relatively mono-disperse nano-order size aggregates of the hydrophobic group-containing polysaccharide exist in water as a dispersion which is colorless and transparent in appearance and does not form any cloud or precipitate, even after allowing to stand for a long period of time, leaving an appearance of an aqueous solution in the human eye.

By causing a hydrophobic group-containing polysaccharide to swell in water and agitating the resulting swollen dispersion using, for example, a homomixer, a turbid dispersion is obtained. In such a turbid dispersion, a part of the hydrophobic group-containing polysaccharide forms

aggregates of a size of nano-order, while there are at the same time some which are present as lumps of various sizes without forming such aggregates. When a turbid liquid, in which lumps of sizes greater than nano-order size are present, is used for a medicinal material, for example, as a material in a drug delivery system (DDS) for intravenous administration, thrombus may be formed due to the above-mentioned lumps. When, on the other hand, the colorless transparent liquid in which the hydrophobic group-containing polysaccharide is present, forming aggregates of uniform nano-order size, is used therefor, there is no fear of thrombus formation. Therefore, there is a demand for aggregates of a hydrophobic group-containing polysaccharide which are dissolved (dispersed in a colorless transparent state) in water, in order to use the hydrophobic group-containing polysaccharide as, for example, a medicinal material for building up a conjugate with a varying kind of drug or protein.

B' cont'd

In the past, processes have been known for forming hydrophobic group-containing polysaccharides into aggregates, for example, 1) a process in which the hydrophobic group-containing polysaccharide is dissolved in dimethyl sulfoxide (DMSO) under a dilute condition and the resulting solution is then dialyzed against water and 2) a process in which the hydrophobic group-containing polysaccharide is caused to swell in water and the resulting swollen dispersion is then treated by ultrasonication {Macromolecules,. 3062 (1993); WO 98/09650}.

It is, however, quite difficult to prepare such aggregates of a hydrophobic group-containing polysaccharide industrially in large scale by the above-mentioned processes of the prior art. Firstly, for example, in the dialysis process of the prior art, there are problems in that 1) a dialysis arrangement capable of large scale treatment is required, 2) a huge amount of water is necessary and 3) a

prolonged period of time is required for the treatment. Secondly, in the process by ultrasonication of the prior art, there are problems in that 1) the throughput of one single treatment is lower, 2) deviation between treating lots is large, since control of sonication efficiency and of sonication time is difficult and monodisperse aggregates are not able to be obtained steadily and 3) probable contamination with, for example, fractured metal fragments may occur due to deterioration of the sonication tip.

With the background described as above, a large scale preparation of aggregates of a hydrophobic group-containing polysaccharide is difficult by the processes with dialysis and ultrasonication of the prior art. Therefore, a more simple and convenient process for forming aggregates of a hydrophobic group-containing polysaccharide is expected. However, no technique of forming aggregates of a hydrophobic group-containing polysaccharide has hitherto been known other than the above-mentioned processes of dialysis and ultrasonication.

B' cont'd

While a homogenizer is used for emulsifying oils in water, no practical experience has heretofore been known in which a homogenizer is used for forming aggregates of a hydrophobic group-containing polysaccharide.

The object of the present invention is to obviate the problems in the prior art described above and to provide a process for forming aggregates of a hydrophobic group-containing polysaccharide, in which the deviation between treating lots and the contamination by impurities are eliminated and which can afford to prepare uniform aggregates of a hydrophobic group-containing polysaccharide steadily in a simple and convenient way within a brief time and in a large scale.

DISCLOSURE OF THE INVENTION

The inventors reached from their sound researches the knowledge that aggregates of a hydrophobic group-containing polysaccharide can be obtained in a simple and convenient way within a brief time in large scale by dispersing a swollen liquor of the hydrophobic group-containing polysaccharide using a high pressure homogenizer, whereby the present invention has been completed. Thus, the present invention consists in the process for forming aggregates of a hydrophobic group-containing polysaccharide as given below:

(1) A process for forming aggregates of a hydrophobic group-containing polysaccharide in water, comprising

Please replace the BRIEF DESCRIPTION OF THE DRAWINGS section, beginning on page 8, line 4, with the following rewritten section.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of Example 1-1 in graphs, each in a chart of the results of SEC analyses of a pullulan-cholesterol derivative (CHP) before and after the treatment by a high pressure homogenizer. Figs. 1(a) and 1(b) are each a chart of analysis results of SEC before and after the treatment of the CHP by the high pressure homogenizer, respectively. The ordinate represents the strength (dimensionless) of the differential refractometer (the same applies to those in the following).

Fig. 2 shows the results of Examples 1-2 to 1-5 in graphs, wherein Figs. 2(a), 2(b), 2(c) and 2(d) are each a chart of analytical results of SEC after the treatment by high pressure homogenizer for Examples 1-2, 1-3, 1-4 and 1-5, respectively.

Fig. 3 shows the results of Comparative Example 1 in a graph, a chart of the results of SEC analysis after the dialysis.

Fig. 4 shows charts of the results of SEC analyses of pullulan (of a molecular weight of 108,000) and of CHP. Figs. 4(a), 4(b) and 4(c) are each a chart of the results of SEC analysis, for the pullulan (molecular weight of 108,000), for the CHP and for the aggregates of the CHP, respectively.

Fig. 5 is a chart of the results of SEC analysis of CHP (a concentration of 0.2% by weight) after ultrasonication for a predetermined period of time.

B2 concl'd

Fig. 6 shows the results of Comparative Example 2 in graphs, namely, charts of the SEC analysis results of a CHP after an ultrasonication treatment and after a treatment by a high-pressure homogenizer, respectively. Figs. 6(a) is a chart of the results of SEC analysis of the CHP after the ultrasonication treatment. Fig. 6(b) is a chart of the results of SEC analysis of the ultrasonicated liquor of Fig. 6(a) after it is treated by the high-pressure homogenizer.

✓

Please replace the paragraph beginning on page 9, line 15, and ending on page 9, line 27, with the following rewritten paragraph.

B3

While there is no special limitation for the hydrophobic group-containing polysaccharide to be employed according to the present invention, so long as it has hydrophobic groups, the following hydrophobic group-containing polysaccharides are preferred. Thus, preference is given to polysaccharides having -XH groups (wherein X denotes an oxygen atom or a nitrogen-containing group represented by NY, with Y standing for a hydrogen atom or a hydrocarbon group of 1 - 10 carbon atoms), wherein 0.1 - 10, preferably 0.1 - 6, -XH groups per 100 monosaccharide units constituting the polysaccharide are replaced by one or more hydrophobic groups represented by the formula (1) given above.

Please replace the paragraphs beginning on page 14, line 9, and ending on page 19, line 21, with the following rewritten paragraphs.

The amount of water to be used in the process step [1] may favorably be 30 - 10,000 times by weight, preferably 100 - 1,000 times by weight, of the hydrophobic group-containing polysaccharide. If this amount is short of 30 times by weight, the hydrophobic group-containing polysaccharide may become an unfavorably gelled state. If this amount exceeds over 10,000 times by weight, the efficiency of forming aggregates will become unfavorably decreased. While there is no special restriction as to the water temperature for effecting swelling, a temperature of 0 - 100 °C, preferably 10 - 50 °C, may be favorable.

The resulting swollen dispersion may favorably be brought to the subsequent process step [2] after having been stirred by a stirrer. As the stirrer to be employed, a magnetic stirrer, a homomixer or the like may be exemplified. Among them, preference is given to the homomixer. While there is no special limitation for the revolution rate, stirring duration and so on of the stirrer, a revolution rate of 100 - 15,000 rpm and a stirring duration of 30 seconds to 180 minutes may be favorable. The dispersion resulting from stirring of the swollen dispersion is present as a turbid liquid, which gives birth to deposition of precipitates after standing for a while.

The homogenizer to be employed in the process step [2] should be capable of dispersion-treating the swollen dispersion from the process step [1] under a pressure of 9.8 - 490 MPa (100 - 5,000 kgf/cm^2), preferably 98 - 294 MPa (1,000 - 3,000 kgf/cm^2). For such a homogenizer, a commercial high pressure homogenizer may be employed. A high pressure homogenizer is a device for attaining emulsification of microdispersion of a liquor by generating shearing forces,

impingement momentums and cavitation by the aid of a high pressure.

When such a high pressure homogenizer is used, aggregates of the hydrophobic group-containing polysaccharide can be formed, specifically, in the following manner. First, the swollen dispersion is pressurized at a pressure mentioned above and the so-pressurized swollen dispersion is spouted from an orifice into a chamber to cause cavitation (pressure drop). The spouted swollen dispersion is thereby accelerated and caused to bring about intense collisions of domains of the swollen dispersion with each other in the chamber and with the walls of the chamber. By the thereby generated impingement momentums and shearing forces, the hydrophobic group-containing polysaccharide is dispersed finely in the dispersion to build up aggregates thereof. The so-obtained treated liquor is present as a transparent colorless liquid which is a dispersion (expressed in the following sometimes as aqueous solution) not subject to the occurrence of turbidity or precipitation after a prolonged standing period.

B1 cont'd

The dispersing treatment using a high pressure homogenizer may be effected only once or in two or more repetitions. The treatment with high pressure homogenizer may be carried out in a batchwise or continuous operation. While the number of repetitions of the high pressure homogenizer treatment may vary considerably depending on, for example, each specific hydrophobic group-containing polysaccharide, the degree of substitution with the hydrophobic group, the concentration in the aqueous dispersion and the pressure of the high pressure homogenizer treatment, a stable and relatively monodisperse aggregate may be obtained usually by five repetitions, though not affirmable. For example, in the case where the hydrophobic group-containing polysaccharide is a pullulan-cholesterol derivative with a cholesterol-substitution degree of 1.2 cholesterol groups per 100 monosaccharide units, the concentration in the aqueous

dispersion is 0.2% by weight and the pressure on the high pressure homogenizer treatment is 98 MPa (1,000 kgf/cm^2), a stable aggregate without suffering from the occurrence of turbidity or precipitation can be obtained by repeating the dispersing treatment by the high pressure homogenizer three times.

Concrete examples of the high pressure homogenizer which can be used in the process according to the present invention include MICROFLUIDIZER (of the firm Microfluidex, trademark), MICROFLUIDIZER (of Mizuho Kogyo K.K., trademark), DeBEE 2000 (trademark, supplied from Q.P. Corp.) and APV GAULIN (trademark, of APV Rannie, Inc.).

While there is no special limitation as to the temperature of the swollen dispersion during the dispersing treatment by a homogenizer, a temperature in the range from 0 to 100 °C, preferably from 10 to 50 °C, may be favorable.

By performing the dispersing treatment using a homogenizer, a monodisperse aggregate can be formed. The resulting monodisperse aggregate, namely, the aggregate of the hydrophobic group-containing polysaccharide obtained by the process according to the present invention, has usually an aggregate particle size in the range from 10 to 30 nm and a number of associations of the hydrophobic group-containing polysaccharide in the aggregate in the range from 3 to 20. Here, the particle size and the number of associations refer both to the average value. The resulting treated dispersion is a colorless transparent aqueous solution which will not become turbid nor bring about precipitation after a prolonged standing period. Here, the monodisperse aggregate will not be formed by simply agitating the swollen dispersion by a stirrer, such as a magnetic stirrer or homomixer. The swollen dispersion keeps its turbid state and will not turn into a colorless transparent state even though the revolution rate of the stirrer is increased or the stirring is continued for a prolonged period of time.

The aggregate of the hydrophobic group-containing polysaccharide formed by the process according to the present invention can be separated in a form of a solid matter by drying the aggregate, after it has been formed, by means of, for example, freeze-drying. From this solid matter, a colorless transparent aqueous solution of the aggregate in the state before the freeze-drying can be restored by adding water to the solid matter.

The aggregate of the hydrophobic group-containing polysaccharide formed by the process according to the present invention can be used as a medicinal material, such as a coating material for coating a drug carrier enclosing therein a drug. Thus, it can be used as a coating material for coating a drug carrier made of, for example, a liposome, microcapsule, microsphere, O/W emulsion or erythrocyte ghost. Here, the aggregate of the hydrophobic group-containing polysaccharide obtained by the process according to the present invention can be used securely as a medicinal material and for preparing a drug carrier of a stable quality, since the so-obtained aggregate is a homogeneous product and has no quality deviation between production lots nor contamination by impurities. The aggregate of a hydrophobic group-containing polysaccharide obtained by the process according to the present invention can also be utilized as a surfactant, thickening agent and a raw material for cosmetics.

B4 D cont'd

In the process according to the present invention, it is possible to employ a mixture of a hydrophobic group-containing polysaccharide with one or more polysaccharides having no hydrophobic group (i.e. those before introduction of hydrophobic group therein) and/or one or more medicaments and/or one or more proteins, instead of using the hydrophobic group-containing polysaccharide solely. Hereby, the possibility of extension of application field, for example, in the drug delivery system (DDS), may be prospective.

As described above, aggregates of a hydrophobic group-containing polysaccharide can securely be formed in a homogeneous quality steadily within a brief period of time, in a large scale and in a simple manner, by the process according to the present invention without suffering from quality deviation between production lots and from contamination by impurities, since the process is performed by a dispersing treatment of a swollen dispersion of the hydrophobic group-containing polysaccharide using a homogenizer under a pressure within a specific range.

Please replace the paragraph beginning on page 20, line 25, and ending on page 21, line 14, with the following rewritten paragraph.

An eggplant type 1-liter flask was charged with 25 grams (0.065 mol) of cholesterol and thereto were added 300 ml of toluene to dissolve it, whereto 17 ml (0.12 mol) of triethylamine were added. To this, 161 grams (0.96 mole, 14.8 eq.) of hexamethylene diisocyanate dissolved in 300 ml of toluene were added to cause a reaction at 80 °C for 6 hours under a nitrogen atmosphere. After termination of the reaction, toluene and the excess amount of hexamethylene diisocyanate were removed by reducing the pressure. The resulting yellowish oily residue was allowed to stand overnight at room temperature to cause precipitation of pale yellow crystals. The crystals were taken out and about one liter of hexane was added thereto, whereupon the mixture was shaken vigorously and, then, the supernatant liquid was removed by decantation. This washing procedure was repeated four times, whereupon the crystals were dried under a reduced pressure at room temperature for three hours, whereby N-(6-isocyanatohexyl)cholesteryl carbamate represented by the following formula (4a) was obtained.

Please replace the paragraph beginning on page 21, line 18, and ending on page 22, line 14, with the following rewritten paragraph.

In an eggplant type flask of 300 ml capacity, there were charged 3.48 g (12.9 mmol) of stearyl alcohol and thereto were added 50 ml of toluene to dissolve it, whereto 2.04 g (25.8 mmol) of pyridine were further added. To this mixture, there were added 30 g (178 mmol, 14.8 eq.) of hexamethylene diisocyanate dissolved in 50 ml of toluene and the resulting mixture was subjected to reaction at 80 °C under a nitrogen atmosphere for about 3 hours. After termination of the reaction, toluene and the excess hexamethylene diisocyanate were removed under a reduced pressure, whereby pale yellow crystals were formed. The crystals were taken out, whereto about one liter of hexane was added and the mixture was shaken vigorously, whereupon the supernatant was removed by decantation. This washing procedure was repeated four times, whereupon the product was dried under a reduced pressure for three hours at room temperature. Hereby N-(6-isocyanatohexyl)stearyl carbamate represented by the following formula (8) was obtained:

Please replace the paragraphs beginning on page 22, line 19, and ending on page 23, line 30, with the following rewritten paragraphs.

A hydrophobic group-containing polysaccharide was synthesized according to the method of Akiyoshi et al {Macromolecules,. 3062 (1993)}. Thus, an eggplant type flask of 1 liter capacity was charged with 40 g (248 mmol as anhydrous glucose unit) of a pullulan (a product of Wako Pure Chemical Industries, Ltd.; average molecular weight: 108,000) and 420 ml of dimethyl sulfoxide (sometimes abbreviated as

DMSO) and the mixture was agitated at 80 °C under a nitrogen atmosphere to dissolve it. To this solution, a solution of 1.78 g (3.21 mmol) of N-(6-isocyanatohexyl)cholesteryl carbamate synthesized in Synthesis Example 1-1 dissolved in 32.4 ml (0.40 mol) of pyridine was added and the mixture was subjected to reaction at 90 °C for 1.5 hours.

After termination of the reaction, dimethyl sulfoxide was removed by reducing the pressure and the resulting oily residue was dropped into 6 liters of acetone to form a precipitate which was purified. After removal of the supernatant, 4 liters of acetone were added to the resulting precipitate and the mixture was allowed to stand overnight at room temperature. The precipitate was collected by filtration and dried under a reduced pressure. The so-obtained solids were dissolved in dimethyl sulfoxide and the solution was charged in a dialysis bag (Spectra/Por3, a product of the frim Spectropor; a fractionating molecular weight of 3,500) and was subjected to a dialysis against distilled water for one week. 1.5 liters of the resulting polymer solution were treated by freeze-drying in an ordinary manner, whereby a pullulan-cholesterol derivative (abbreviated hereinafter sometimes as CHP) represented by the following formula (7a) was obtained. By calculating the proportion of introduction of the cholesteryl groups into the pullulan in the CHP from the integration value of the ^{1}H-NMR spectrogram of CHP, it was determined that the proportion of substitution with cholesteryl group in the pullulan-cholesterol derivative (CHP) represented by the formula (7a) was about 1.3 groups per 100 monosaccharide units.

B7 concld

Please replace the paragraph beginning on page 24, line 8, and ending on page 24, line 16, with the following rewritten paragraph.

In the same manner as in Synthesis Example 2, except that a commercial mannan (a product of the firm Sigma) having an average molecular weight of about 85,000 was used in place of the pullulan, a mannan-cholesterol derivative (in the following, sometimes abbreviated as CHM), in which about 2.3 cholesteryl groups are introduced per 100 monosaccharide units, represented by the following formula (7b) was synthesized.

Please replace the paragraph beginning on page 25, line 14, and ending on page 26, line 19, with the following rewritten paragraph.

There were added 1,000 ml of water to 2 grams of the CHP obtained in Synthesis Example 2 to cause the CHP to swell at a temperature of 60 °C for 2 hours (CHP concentration = 0.2 % by weight). The resulting swollen dispersion was then stirred using a homomixer (5,000 r.p.m.) for 5 minutes. The appearance of the dispersion at this occasion was white turbid. The so-stirred swollen dispersion of 20 ___°C was subjected to a homogenization by causing the dispersion to spout out of an orifice under a pressure of 98 MPa (1,000 kgf/cm^2) using MICROFLUIDIZER (trademark, a high pressure homogenizer Model M-110Y of the firm Mizuho Kogyo K.K.) into a chamber in order to disperse it. This homogenization treatment was repeated twice. The herein used MICROFLUIDIZER had a treating capacity of about 500 ml/min. and the time required for two repetitions of the homogenization treatment was about 5 minutes. The resulting treated liquor had a colorless and transparent appearance. For this aqueous solution, the particle size and the number of associations were determined by the methods indicated above. The results are summarized in Tables 1 and 2.

Please replace the paragraph beginning on page 26, line 29, and ending on page 27, line 12, with the following rewritten paragraph.

Then, the resulting aqueous solution of the CHP aggregates was subjected to a freeze-drying, whereby the aggregates of the CHP were isolated as a white solid matter. To this solid matter, water was added so that a concentration of 0.2 % by weight would be reached, whereupon the mixture was allowed to stand at room temperature for three hours in order to restore an aqueous solution. The restored solution was colorless and transparent. For the aqueous solution of the CHP aggregates before the freeze-drying and for the restored solution, SEC analyses were carried out, whereby it was recognized that there was no distinction in the chart curve between both the solutions and confirmed that both were identical.

Please replace the paragraph beginning on page 27, line 23, and ending on page 27, line 30, with the following rewritten paragraph.

By performing the freeze-drying in the same manner as in Example 1-1, the aggregates in each Example were isolated in a form of white solid matter. For the aqueous solution of the aggregates before and after the freeze-drying, comparison was carried out as in Example 1-1, whereby it was recognized that there was no distinction therebetween and confirmed that both were identical.

Please replace the paragraphs beginning on page 32, line 28, and ending on page 33, line 29, with the following rewritten paragraphs.

From the results given above, it is seen that a sufficient formation of aggregate was not able to be attained using an ultrasonication, whereas the process as shown in the Examples using a high pressure homogenizer was able to attain formation of an aggregate easily.

It is also seen that aggregates exhibiting a narrower molecular weight distribution were formed in Examples 1-1 to 1-6 in which a high pressure homogenizer was used, as compared with the results of Comparative Example 1 in which dialysis was employed and of Comparative Example 2 in which an ultrasonic wave treatment was used. It is further seen that aggregates of a hydrophobic group-containing polysaccharide can be formed within a brief time in a simple and convenient manner in large amount by the process according to the present invention, since inventive Example 1-1 showed a productivity of 2 grams in a treating time of 5 minutes, whereas Comparative Example 1 using dialysis showed a productivity of 2 grams in a treating time of 4 days and Comparative Example 2 using ultrasonication showed a productivity of 2 grams in a treating time of more than two hours.

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INDUSTRIAL APPLICABILITY

The aggregates of a hydrophobic group-containing polysaccharide formed by the process according to the present invention can be utilized as a coating material for coating drug carriers encapsulating therein drugs. For example, it can be used as the coating material for coating drug carriers, such as liposome microcapsules, microspheres, O/W emulsions and erythrocyte ghosts.

IN THE CLAIMS

Please cancel Claims 1-6.

Please add new Claims 7-14 as follows.